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DELAY AND QUADRIPARTITION IN SEA URCHIN EGGS INDUCED BY SHORT EXPOSURE TO 2-MERCAPTOETHANOL

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The effects of 2-mercaptoethanol (ME), an inhibitor of cell division in a variety of cells (Mazia, 1958; Limbosch-Rolin and Brachet, 1961; Fautrez and Fautrez-Firlefyn, 1963; Fotakis, 1963; Limbosch-Rolin, 1963; Meeker, 1964; Mazia and Zeuthen, 1966), have been studied extensively by Mazia and his coworkers using echinoderm eggs. They reported that ME blocks cleavage reversibly if treatment is initiated before the "point of no return" which corresponds approximately to metaphase, and that the delay in cleavage is equal to the time spent in ME (Mazia, 1958; Mazia and Zimmerman, 1958). In addition, they found that DNA synthesis continues (Bucher and Mazia, 1960) and that the level of respiration is high enough to support cleavage (Mazia and Zimmerman, 1958) during the ME treatment. The mitotic apparatus (MA) can be isolated during the ME treatment, although the fibrous structures of MA seem disrupted with phase contrast microscopy (Mazia and Zimmerman, 1958). Pressure studies of Zimmerman (1964) revealed that ME also weakens cortical gel strength.

It was concluded that ME arrests cleavage at the time of exposure to ME, because the delay is equal to the duration of the treatment, and that this arrest is somehow linked to the abnormality in structure of the isolated MA.

In addition to the synthesis of DNA and ATP, the first cleavage of echinoderm eggs appears to require protein synthesis (Hultin, 1961; Zotin, Milman and Faustov, 1965; Young, Hendler and Karnofsky, 1969). In order to further elucidate the mechanisms of ME action on cleavage, it is necessary to obtain more detailed information on the response of cells to ME during the cleavage cycles and on the relationship between protein synthesis required for division and the effects of ME. These problems have been investigated in the present study.

The phenomenon of quadripartition (QP), a direct division of one cell into four, occurs during recovery from the prolonged blockage of cleavage by ME. QP involves the generation of a tetrapolar mitotic apparatus from a bipolar one (Mazia and Zimmerman, 1958). Mazia, Harris and Bibring (1960) established that each mitotic center is duplex in nature, *i.e.*, there are four potential centers at metaphase, and that duplication of centers occurs at about telophase. They further suggested that ME is capable of blocking duplication of the centers while permitting the "moving-apart" of existing centers according to the regular mitotic schedule. These conclusions were based on several observations. First, the quadripartitioned cells skipped the next cleavage although other mitotic events including the formation of a monopolar mitotic spindle occurred. Secondly, QP could not be induced by the ME treatment unless a certain time had elapsed after fertilization and unless exposure to ME was of such duration that it extended past the division of controls and the ME treated cells divided after the second divi-

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sion of controls. The inhibition of duplication of mitotic centers by ME was verified by Went (1966) using a more unambiguous agent (benzimidazole) for assaying the number of mitotic centers.

In the present study, new observations have been made that may modify the present hypothesis regarding QP. The capacity of androgenic merogones to undergo QP was also examined.

Materials and Methods

Arbacia punctulata eggs, obtained by electrical stimulation, were shed directly into a beaker filled with filtered sea water (Harvey, 1952). *Lytechinus variegatus* eggs were shed by placing a few drops of 0.55 *M* KCl into the test (Palmer, 1937). All eggs were washed twice.

The following procedures were used for the short exposure experiments. Eggs were inseminated and washed about a minute later to remove excess sperm. At 3–5 minute intervals after insemination, 1 ml of egg suspension, containing 400–500 eggs, was mixed with 1 ml of 0.2 *M* ME dissolved in sea water. After the desired period of exposure (5–80 minutes depending upon the experiments), eggs were washed by centrifugation or by dilution (a dilution factor of 1:10,000). Both centrifugation and dilution methods gave essentially the same results. The controls were treated similarly. The time when approximately 50% of the eggs had cleavage furrows was estimated visually. In order to determine the percentage of quadripartition (QP), 200 cells were examined before the onset of the second cleavage. Data obtained from asynchronously dividing cells were discarded.

After several preliminary experiments, 0.1 *M* ME was chosen because this was the lowest concentration that gave consistent results. For example, 0.7 *M* did not block cleavage consistently in both *Lytechinus* and *Arbacia* eggs.

In some experiments ME-treated eggs were further treated with puromycin, a specific inhibitor of protein synthesis (Nathans, 1964). Each aliquot of eggs was quickly washed twice by dilution (for 1 minute) and 0.2 ml of egg suspension containing about 200 eggs was mixed with 0.2 ml of 1×10^{-3} *M* puromycin on a depression slide. The slide was placed in a petri dish with filter papers moistened with sea water to prevent evaporation. In experiments where eggs were first pulsed with puromycin and then treated with ME, 1 ml of 1×10^{-3} *M* puromycin was mixed with 1 ml of fertilized egg suspension, washed three times after a 15 minute period of incubation and then subsequently, at different times, exposed to ME for 20 minutes.

Androgenic merogones were obtained by inseminating the enucleate fragments of eggs obtained by cutting (for detailed method see Rustad, Yuyama and Rustad, 1970). Briefly, unfertilized eggs were cut with a thin glass needle into approximately equal halves in a Syracuse dish coated with 0.2% agar. Enucleate and nucleate halves were sorted immediately after each cut, since the half containing a female pronucleus can be determined during the time of cutting. Approximately 30 minutes were required to cut 60 eggs. Extreme caution was taken to prevent polyspermy in fertilizing the cut eggs.

Determination of the incorporation of ^{3}H-leucine was performed in the following manner. Sea urchin eggs were washed twice with sea water filtered through Millipore filters, inseminated, washed and transferred into 10 ml of sea water

containing either ME or puromycin. After 5 minutes, 1 μc/ml ^{3}H-leucine (5.0 c/mmole, New England Nuclear) was added. The reaction was terminated by adding an equal volume of 10% trichloroacetic acid (TCA) and the samples

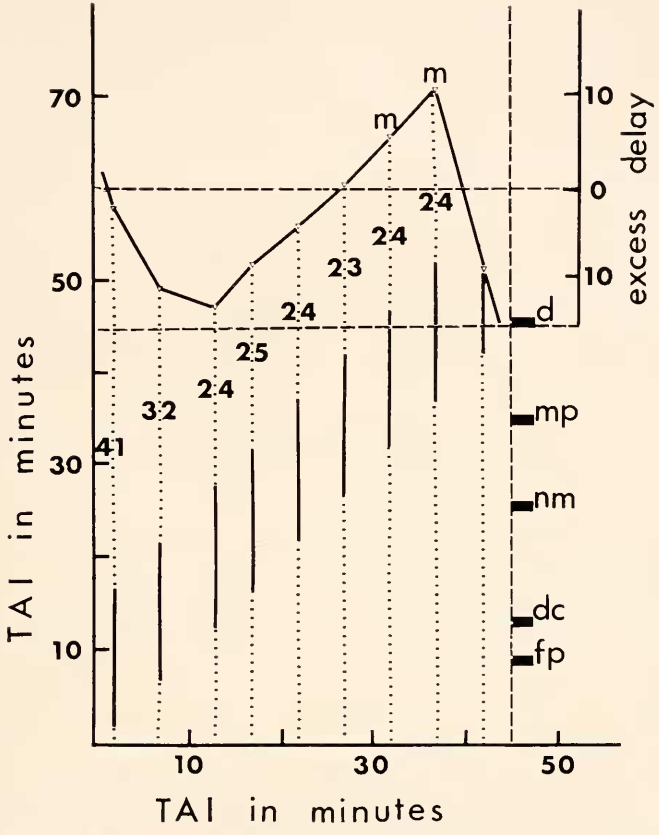

FIGURE 1. Delay-ME exposure diagram of *Lytechinus* eggs. Eggs were fertilized, and treated by ME for 15 minutes, initiated at different times after insemination. Lengths of each vertical line indicate the first cleavage period of *Lytechinus* eggs. Duration of the ME treatment is indicated by the solid line. Numbers associated with each vertical line indicate the recovery period. The mitotic stages of the control eggs are indicated on the right side of the graph. Abbreviations used are: TAI = time after insemination; m = multiple division in some eggs; d = time of control cleavage; mp = metaphase; nm = disappearance of the nuclear membrane; dc = centriole separation; fp = fusion of pronuclei; excess delay = cleavage period of ME treated eggs − cleavage period of control egg − duration of the ME treatment.

were heated for 15 minutes at 90° C. An excessive amount of non-radioactive leucine was added and the samples were kept overnight in the cold room. The samples were filtered through glass fiber filters, washed with 70% ethanol, dried, placed in vials containing toluene and proper fluors and the radioactivity was counted using a Nuclear Chicago Scintillation Counter.

Results

Cleavage delay

In the first series of experiments, *Lytechinus* eggs were inseminated and exposed to 0.1 *M* ME for periods of 15 minutes, starting at various times after fertilization. Typical results are shown in Figure 1. During the first cleavage

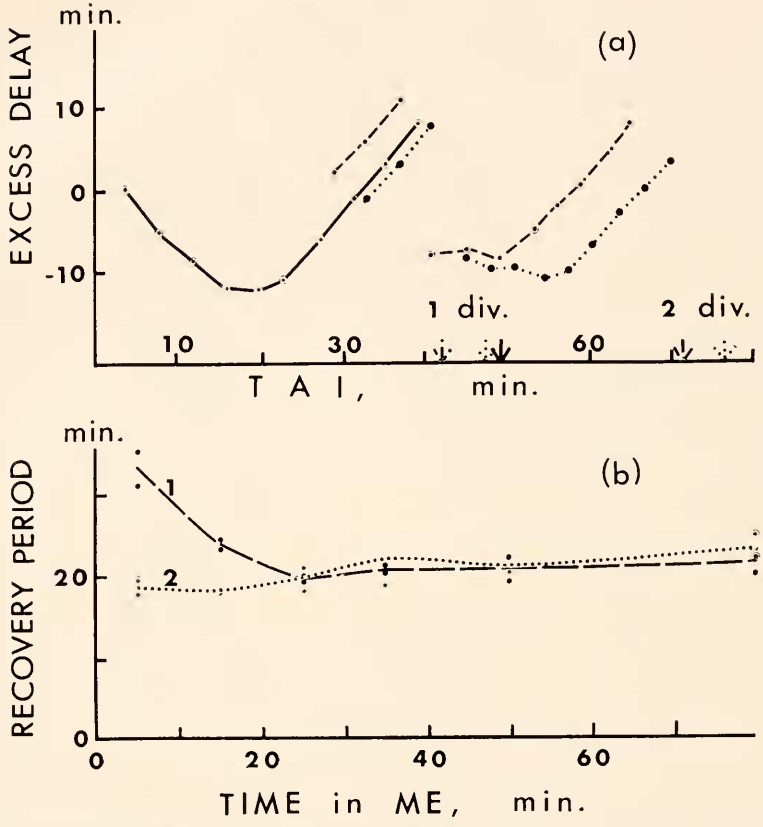

Figure 2. Delay-ME exposure diagram of *Arbacia* eggs. (a) The experimental design is similar to that of Figure 1, although eggs are treated for 20 minutes in this experiment. Different lines indicate separate experiments. (b) Dependence of the recovery period upon the duration of the ME treatment, the treatment was initiated either at the beginning of the streak stage (line 1) or several minutes before metaphase (line 2).

cycle, there was a change in delay, although the exposure period was kept constant. With batches of cells whose first division cycle was 45 minutes, the delay decreased when the treatments were initiated up until 13 minutes after insemination, increased linearly up until 37 minutes and decreased rapidly after this time. During the period when delay increased linearly (the linear delay phase), the interval between the end of exposure and the time of cleavage was constant (Fig. 1). Although the period of the first cleavage cycle varied among different

batches of eggs (45–60 minutes) and although the amount of delay varied with different experiments, the essential pattern of delay was the same in six different experiments. The start of the linear delay phase superficially corresponds to the beginning of the streak stage; division (separation) of the mitotic centers appears to occur about this time (Wilson and Mathews, 1895). The end of the linear delay phase corresponds approximately to metaphase after which sensitivity of cells to ME is rapidly lost. The above experiments established that the delay induced by ME treatment is not constant but fluctuates during the cleavage cycle.

In order to examine whether or not this fluctuation of delay would occur in another genus of sea urchin eggs, the same short exposure experiments were performed with *Arbacia* eggs. The delay pattern of *Arbacia* eggs was essentially the same as that of *Lytechinus* (Fig. 2a). In addition, the linear delay phase was also observed in the second division cycle. Since the linear delay phase reflects the constant period between the end of exposure and th time of cleavage (see Fig 1), it suggests that ME damages a certain event related to cytokinesis and that recovery from this damage takes a constant period of time. In order to examine whether this ME sensitive event is present from the beginning of the streak stage or develops at later stages, the dependence of the recovery period upon the exposure time to ME was studied. Fertilized eggs were exposed to ME for various periods of time starting at two different stages (beginning of the streak stage and immediately before the end of the linear delay phase; *ca.* metaphase). Typical results are shown in Figure 2b. When the ME treatments were initiated at the beginning of the streak stage, the recovery period gradually decreased and then became constant after periods of exposure for about 20 minutes or longer. When the exposure was initiated immediately before the end of the linear delay phase, the recovery period did not depend upon the period of exposure between 5 and 80 minutes.

These observations suggest that the ME sensitive event develops about 20 minutes after the beginning of the streak stage. This time is several minutes before metaphase. At about metaphase, eggs lose sensitivity to the ME treatment. In all experiments the recovery period (an interval between the end of exposure to ME and the time of cleavage) is 20–25 minutes and is longer than the interval between metaphase and cleavage (8–12 minutes).

In the next series of experiments, the effects of temperature on the recovery period were investigated. An Arrenius plot of the data indicated that the energy of activation for the recovery period is 16,400 calories.

In the third series of experiments, the relationship between protein synthesis required for division and the blockage of cleavage by ME was investigated in *Arbacia* eggs. Eggs were exposed to puromycin (5×10^{-4} *M*) immediately after fertilization for a period of 15 minutes. This treatment resulted in approximately 30 minute delays in cleavage; the synchrony of cleavage was not appreciably changed by the treatment. When these puromycin-treated cells were exposed briefly to ME at various times during the cycle, the appearance of the linear delay phase was delayed for approximately 30 minutes (Figure 3). In four separate experiments, there was a precise correlation between the delay of cleavage due to the puromycin treatment and the delay of the appearance of the linear delay

phase. These results indicate that the ME sensitive event arises only after the protein synthesis required for division.

Arbacia eggs were continuously treated with puromycin. When the treatments were initiated at different times after fertilization, cleavage was blocked up until about 10 minutes after insemination. After this period, cleavage was delayed up until the beginning of the streak stage (delay decreased rapidly during this period) and then the first cleavage was no longer affected by the puromycin treatment. These findings confirmed the observation of Rustad and Burchill (1966). When the cells were treated with ME for 20 minutes during the puromycin sensitive period and then incubated immediately with puromycin (5×10^{-4} *M*), it was found that sensitivity to puromycin is markedly reduced after the ME treatment. This

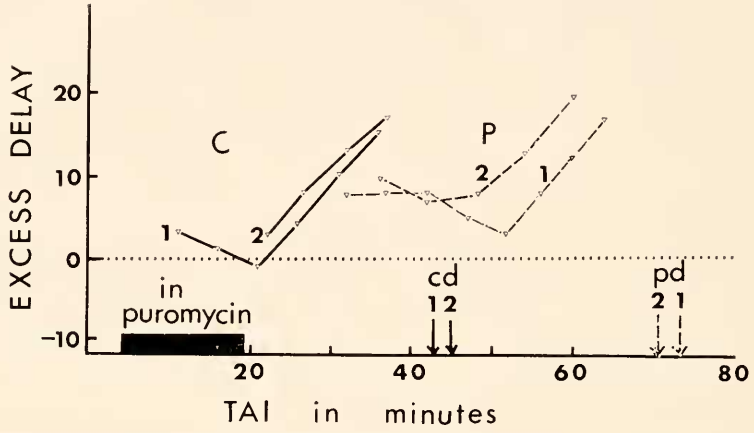

FIGURE 3. Delay-ME exposure diagram of the puromycin treated cells. The delay patterns of *Arbacia* eggs treated by ME alone (C) are compared with those pulsed with puromycin for 15 minutes before the ME treatment (P). Lines with the corresponding numbers represent experiments performed with the same batches of eggs. Abbreviations used are: cd = cleavage time of controls; cp = cleavage time of puromycin treated controls.

suggests that protein synthesis required for division continues during the ME treatment. In order to verify this point, eggs were incubated with ^{3}H-leucine (1 μc/ml) for a period of 30 minutes in the presence of ME (0.1 *M*) or puromycin (5×10^{-4} *M*). The hot TCA insoluble radioactivity was determined. The average counts per minute (from two separate experiments) were:

Control 8495; ME treated 6714; puromycin treated 1317.

During the linear delay phase, no additional delay was observed with eggs which were treated with puromycin after the ME treatment, suggesting that recovery from the ME treatment does not require protein synthesis.

Went (1962) reported that dithiodiglycol (DTDG), an oxidized product of ME, solated the mitotic apparatus and inhibited cleavage. Since the effective concentration of DTDG (1×10^{-3} *M*) was much lower than that of ME (1×10^{-1} *M*), there was a possibility that the effects of ME were due to DTDG contamination in the ME solution, as contamination by 1% DTDG in ME may produce the effect. The effects of DTDG (1×10^{-3} *M*) on the cleavage of *Arbacia* eggs

were studied. With DTDG the sharp transition of sensitivity which was the characteristic of the ME effect, was not observed during the first cleavage cycle. Eggs exposed to DTDG at later stage of the cycle showed a characteristic abnormality of furrow formation (wrinkled all over the egg surface). After short exposures to DTDG (20 or 30 minutes), eggs divided so asynchronously that it was impossible to estimate the 50% division time precisely. These observations rule out the possibility that the effects of ME observed in the present study were due to contamination of the ME solution.

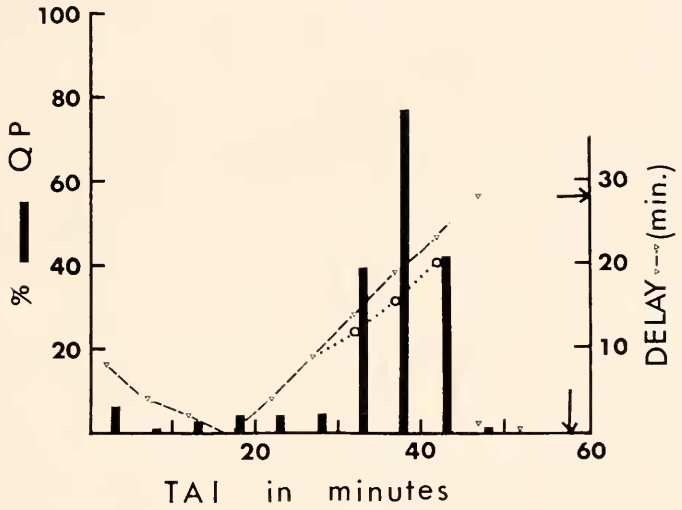

FIGURE 4. Early quadripartition in *Lytechinus* eggs. Experimental design is similar to that of Figure 1, although eggs were treated by ME for 20 minutes. Triangles = cleavage time of bipartitioned cells; circles = cleavage time of quadripartitioned eggs. Solid bars indicate the percentage of QP; arrows = cleavage time of controls.

Quadripartition

Frequencies of quadripartition (QP) varied widely in different experiments. Typically, a majority of cells underwent QP only after prolonged exposure to ME. Sometimes, however, high frequencies of QP were observed in *Lytechinus* eggs after a 15–20 minute exposure to ME. The result of one of these observations is shown in Figure 4. In this experiment, fertilized eggs were exposed to ME for a period of 20 minutes starting at different times after insemination. With a 58 minute cell cycle, frequencies of QP increased markedly (from 4% to 39%), starting at 32 minutes after insemination, reached a peak (77%) at 37 minutes and decreased (42%) at 42 minutes. At 47 minutes, some cells were no longer blocked and no significant percentage of QP was observed. In the case where 39% showed QP, cells were removed from ME 6 minutes before the onset of the first cleavage (not a single cell in the control started to divide at this time), and they divided 18 minutes before the second cleavage. In the case where 77% showed QP, cells were removed 1 minute before the first cleavage and they divided

15 minutes before the second cleavage. This phenomenon will be referred to as "early quadripartition." These observations demonstrate that it is not necessary to treat cells for a prolonged period of time to induce quadripartition. The majority of cells undergoing QP divided about 5 minutes earlier than bipartitioned cells. The phenomenon of "early QP" was not observed in *Arbacia* eggs, however.

In the next series of experiments, the role of the female pronucleus on QP was investigated. Whole eggs, encleate halves and nucleate halves of *Arbacia* eggs were inseminated and treated with ME for 30 minutes starting 30 minutes after the insemination. QP occurred in androgenic merogones in two separate experi-

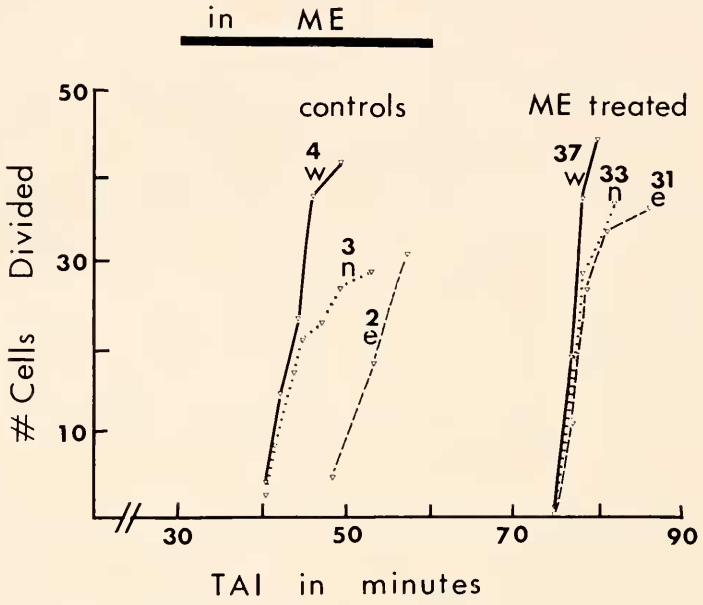

FIGURE 5. QP in the androgenic merogones of *Arbacia* eggs. Numbers associated with each line indicate the number of eggs undergoing QP; W = whole eggs; n = nucleated halves; e = enucleated halves.

ments, indicating that the female pronucleus and half of the cytoplasm are not required for QP (Fig. 5).

The first cleavage of androgenic merogones obtained by cutting is always delayed for 5–15 minutes compared with diploid controls (Rustad *et al.*, 1970). It was found that this characteristic delay disappeared after the ME treatment (Fig. 5).

DISCUSSION

Cleavage delay

Delays induced by a short exposure to ME initiated at various times after fertilization depend upon the stage during the cleavage cycles. During the first and the second cycles, there exists a linear delay phase. This phenomenon occurs

because the interval between the end of exposure to ME and the time of cleavage (recovery period) is constant. This indicates that ME damages a certain specific event required for cleavage and that recovery from this damage takes a constant period of time. The previous observation that the delay in cleavage is equal to the duration of exposure (Mazia, 1958) can be explained by assuming that the ME treatment was initiated at a certain stage such that the recovery period was equal to the interval between the time of the initiation of exposure and the time of control cleavage.

Although the existence of the linear delay phase can also be explained by an alternative possibility that ME merely arrests cleavage at a certain stage during the cycle, this interpretation is unlikely because of the following reasons. First, the recovery period (20–25 minutes in *Arbacia*) is much longer than the interval between the time when eggs become insensitive to ME and the time of cleavage (8–12 minutes). This longer duration of the recovery period cannot be explained by mere diffusion of ME from eggs, for the energy of activation of the recovery period is much greater than the diffusion process in general. In addition, it is known that the temperature coefficient of the processes between metaphase and cleavage is low (Ephrussi, 1933). Secondly, the effects of ME are not completely reversible; cells often divide into four after the ME treatment (Mazia and Zimmerman, 1958).

The results of studies investigating the dependence of the recovery period upon the duration of the ME treatment, indicated that the ME sensitive event develops several minutes before the transition point; the point at which eggs lose their sensitivity to ME. Thus the ME sensitive event must be present only briefly (for about 5 minutes) during the cleavage cycle. This duration is much shorter than the period during which MA is visible in the cell.

The furrowing itself is not blocked by ME, for the transition point occurs several minutes prior to the onset of the furrowing (Mazia, 1958). In addition, furrows induced by pressure centrifugation are not blocked by ME (Zimmerman, Murakami and Zimmerman, 1968). Thus, the mechanisms of the division block by ME must be sought in some events which occur prior to the furrowing.

Fluctuation of protein bound -SH groups in dividing sea urchin eggs appears to be linked to cytokinesis (Rapkin, 1931; Sakai and Dan, 1959). The fact that the pattern of delay induced by ME and by ether (Swann, 1954) are very similar and the fact that ether freezes the change of -SH groups in cortical proteins (Sakai, 1963) suggest that there is a close relationship between the activities of the -SH groups and the action of ME. However, if freezing of the changes in -SH groups is totally responsible for the blockage of division by ME, the recovery period should become progressively shorter. The results of the present experiments contradict this conclusion.

Puromycin inhibits protein synthesis and arrests cleavage at the streak stage in sea urchin eggs (Hultin, 1961). Cycloheximide also blocks cleavage in echinoderm eggs (Young *et al.*, 1969). Puromycin sensitivity is rapidly lost at about the time of the streak stage and the sensitivity period to puromycin is extended in the irradiated eggs (Rustad and Burchill, 1966) in which the streak stage is prolonged (Henshaw, 1940). These observations suggest that the protein synthesis required for cleavage is completed at some time during the streak stage. This

is well before the development of a postulated ME sensitive event. The results of the present experiments revealed that (a) short exposure to puromycin delayed cleavage and postponed the appearance of the linear delay phase for an equal length of time, (b) eggs were not sensitive to puromycin after the ME treatment during the linear delay phase, (c) the ME treatment reduced puromycin sensitivity before the onset of the linear delay phase and (d) the incorporation of radioactive leucine into hot TCA insoluble fractions was not appreciably reduced during ME treatment. These observations suggest that (a) protein synthesis required for division continues during the ME treatment, (b) the ME sensitive event develops only after the synthesis of proteins required for cleavage and (c) recovery from ME treatment does not require protein synthesis.

The importance of MA in determining the orientation and the position of the division furrow in various cells has been discussed by many authors (Kawamura, 1960; Scott, 1960, 1965; Rappaport, 1965; see also Mazia, 1961 for review), although the cortical region of eggs acquires a capacity of autonomous furrowing prior to cytokinesis (Swann and Mitchison, 1953; Hiramoto, 1956. The oriented structure of MA appears disrupted during the ME treatment, but it regains its normal structure rapidly after the eggs are returned to normal sea water. However, cytokinesis does not start immediately (Mazia and Zimmerman, 1958). During the ME treatment, the characteristic birefringence of MA persists (Yuyama, unpublished). At the electron microscope level, the characteristic microtubules of MA are present, although they are arrayed haphazardly (Harris, 1962).

These observations are consistent with the idea that ME blocks cleavage not by destroying the structure of MA, but by affecting the major function of MA—determination of the position of the cleavage furrow in the cortical region. It is conceivable that the hypothetical damage by ME of the original furrowing position is related to quadripartition (QP), since the damage might alter the spacial orientation of the mitotic spindle and distort the animal vegetal axis.

Quadripartition (QP)

Since the position of the furrow is directly related to the structure and the position of MA (Mazia, 1961), it is obvious that the formation of a tetrapolar MA is responsible for QP (Mazia and Zimmerman, 1968). In normally dividing cells, a mitotic center contains two centrioles (de Harven and Bernhard, 1956; Harris, 1961; Costello, 1961), and this structural duplicity is presumably the basis for the formation of four functional poles in QP (Mazia *et al.*, 1960; Went, 1966). The present study has shown that *Lytechinus* eggs which were briefly treated with ME before the first division underwent QP well before the second division of controls. Although this observation is consistent with most of the important concepts developed by Mazia and his coworkers (Mazia *et al.*, 1960; Went, 1966), it appears to demand a slight modification of the hypothesis on QP, since the establishment of four poles occurs well before the normal mitotic schedule. The simplest interpretation would be to assume that ME functionally dissolves connections between the two centrioles in each center so that each centriole becomes independent during the recovery. The independence of each center in the tetrapolar MA seems certain, since the distribution of chromosomes

to each of four blastomeres is random (Bibring, 1962). Recent electron microscopic studies on normally dividing HeLa cells, however, suggested that only one of the two centrioles might participate in spindle tubule formation (Robbins and Jentzsch, 1969). It is conceivable that ME actively causes the premature "splitting" of centrioles, a putative event which permits the members of a pair of centrioles to separate and function independently (Mazia *et al.*, 1960), prior to their duplication.

The induction of QP in androgenic merogones demonstrated that the female pronucleus and half the cytoplasm are not required for QP. This is consistent with the concept that mitotic centers are brought in by sperm after normal fertilization (Boveri, 1900; Dirksen, 1961). Since many agents such as ether (Wilson, 1902; Swann, 1954), benzimidazole (Went, 1966), dinitrophenol, NaN_3 and detergents (Kuno, 1954; Kuno-Kojima, 1960) and butanol (Yuyama, unpublished) can induce multipolar division, the -SH radical of ME is probably not important in inducing QP. ME (and hence -SH) may be unique, however, in that it inhibits the duplication of mitotic centers (Mazia *et al.*, 1960).

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Summary

Eggs of *Arbacia punctulata* and *Lytechinus variegatus* were treated with 0.1 *M* 2-mercaptoethanol (ME) for short periods initiated at different times after fertilization. In both the first and second cleavage cycles, there was a phase during which cleavage delay increased linearly. This linear delay phase reflects the constant period of recovery from damage caused by ME treatment. The ME sensitive event is estimated to be present only briefly (about 5 minutes) immediately prior to the metaphase and appears to be associated with processes that determine the cleavage furrow. This ME sensitive event must develop only after completion of the puromycin sensitive event (protein synthesis required for division), because there is an exact correlation between the puromycin induced delay and delay in the appearance of the linear delay phase.

Lytechinus eggs treated briefly with ME before the first cleavage underwent quadripartition well before the second cleavage of controls. ME, therefore, appears to cause a premature splitting of centrioles prior to their duplication. Quadripartition can also be induced in androgenic merogones, verifying the old concept concerning the role of sperm centrioles in normal cleavage.

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